

Synthesis of silver nanoparticles using curcumin-cyclodextrins loaded into bacterial cellulose based hydrogels for wound dressing applications

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ABSTRACT

Chronic wounds are often recalcitrant to treatment due to high microbial bioburden and the problem of microbial resistance. Silver is a broad spectrum natural antimicrobial agent with wide applications extending to proprietary wound dressings. Recently silver nanoparticles have attracted attention in wound management. In the current study, the green synthesis of nanoparticles was accomplished using a natural reducing agent, curcumin which is a natural polyphenolic compound, well known as a wound healing agent. The hydrophobicity of curcumin was overcome by its microencapsulation in cyclodextrins. This study demonstrates the production, characterisation of silver nanoparticles using aqueous curcumin:hydroxypropyl- β -cyclodextrin complex and loading them into bacterial cellulose hydrogel with moist wound healing properties. These silver nanoparticle-loaded bacterial cellulose hydrogels were characterised for wound management applications. In addition to high cytocompatibility, these novel dressings exhibited antimicrobial activity against three common wound infecting pathogenic microbes *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida auris*.

▪ INTRODUCTION

Microbial colonisation, where is not wanted, can led to severe infections which can result in disability, disease and even death. Wound infections are one of the major factors resulting in impaired healing¹⁻³. Wounds colonised by opportunistic microbes may fail to follow the natural reparative and regenerative stages involved in healing process⁴. In addition uncontrolled infections can prevent the restoration of the anatomical and physiological integrity resulting in chronic non-healing wounds^{5,6}. To prevent infections, modern medicine is dependent on antimicrobial agents such as antibiotics which can either destroy pathogens or inhibit their growth. Unfortunately, the misuse of antibiotics has resulted in the emergence of a number of multiresistant microbes. Therefore antibiotic therapy often proves ineffective in eradicating infections in chronic non-healing wounds³. Owing to the site specific delivery, increased target site concentration, reduced adverse effects, use of agents not suitable for oral and systemic therapy and low incidence of resistance^{7,8}, antimicrobial-loaded wound dressings have attracted wide interest as a concurrent wound management regime.

Hydrogels are one of the promising candidates as wound dressings due to their unprecedented properties including their ability to maintain a moist microclimate at the wound site which is proven to facilitate healing⁹⁻¹¹. A number of natural and synthetic polymeric materials are in use to produce hydrogel dressings^{12,13}. Bacterial cellulose (BC) is an example of a natural biosynthetic cellulose hydrogel which due to its unique properties has been widely considered as a wound dressing¹⁴⁻¹⁸. BC can be produced by several bacteria but *Gluconacetobacter xylinus* (*G. xylinus*) is considered as one of the best for BC production¹⁹. Our group has previously reported the findings on the physicochemical characterisation and suitability of BC hydrogels as

wound dressings²⁰. Although BC does not have inherent antimicrobial activity however, its cross-linked fibre network structure encouraged several research attempts of loading antimicrobials, anti-inflammatory, antioxidants and other healing agents for wound dressing applications^{17,21,22}. In continuation, in the presented study, an investigation has been made to produce biosynthetic hydrogels with healing properties for chronic wound management. Our novel approach is consistent with the concept of the forensic engineering of advanced polymeric materials (FEAPM) which can help to understand the relationships between the structure of the biomaterial used, its properties and behaviour for practical applications²³.

Currently the treatment of microbial infections is severely affected by the emergence of new multiresistant microbes, including bacteria and fungi. Gram-positive *Staphylococcus aureus* has a great ability to develop multiple drug resistances. Similarly, Gram negative *Pseudomonas aeruginosa*, a common opportunistic pathogen shows resistance to most penicillins, cephalosporins and carbapenems. Pathogenic fungi such as *Candida auris* pose a significant health risk to patients suffering from infected wounds and in particular to immunocompromised individuals which can result in candidiasis/candidemia and invasive aspergillosis. Recently a new multidrug resistant strain of *C. auris* was discovered²⁴ and has quickly become a prolific nosocomial pathogen in the UK^{25,26}, with diagnoses on all continents with 33 countries reporting incidents of antifungal resistance. *C. auris* is resistant to most first and second-generation antifungals with 90% of isolates being resistant to fluconazole, 40% resistant to amphotericin and 2% to echinocandins such as caspofungin²⁷⁻²⁹. The concern regarding these pathogens centres around the increasing resistance they are showing towards a very limited pool of effective antimicrobials³⁰⁻³² and hence the focus is on targeted delivery by antimicrobial wound dressings.

Silver is a well-known antimicrobial effective against fungi, yeast and bacteria including several antibiotic resistance strains³³⁻³⁵. The emergence of nanotechnology enabling the production of silver nanoparticles has served a new therapeutic modality. Silver nanoparticles (AgNP) owing to their characteristic broad-spectrum antimicrobial properties have received increased focus in biomedical applications including for wound management³⁶⁻³⁸. Among the several different approaches to synthesise AgNP, the use of natural substances like plant extracts has received wide research consideration due to the safe and eco-friendly procedure^{37,39-41}.

Curcumin (CUR), a natural polyphenol, extracted from turmeric is a well-known wound healing agent with proven antimicrobial, antioxidant and anti-inflammatory effects^{22,42}. In addition to its healing properties, CUR has been used as a reducing and capping agent to produce AgNP⁴³. The main challenge that limits the wider biomedical application of CUR is its hydrophobic nature. Several chemical mediators like dimethyl sulfoxide, dichloromethane, sodium carbonate are employed in AgNP synthesis from CUR⁴⁴⁻⁴⁸. These chemical mediators can have serious cytotoxic effects⁴⁸. Selection of solvent medium, reducing agent and nontoxic stabiliser are the main considerations in green nanoparticle production^{49,50}. The current study reports the production of AgNP following a green chemistry approach using an aqueous solution of curcumin:hydroxypropyl- β -cyclodextrin (CUR:HP β CD). These AgNP produced using CUR:HP β CD (cAgNP) were characterised and loaded in the biosynthetic BC hydrogels to produce hydrogel dressings. To the best of our knowledge, the protocol presented herein to produce cAgNP is novel and this is the first time the production of cAgNP-loaded BC has been reported. This report presents the unique combination of silver nanoparticles with curcumin in the biosynthetic BC to obtain the hydrogels for wound management applications. This study

investigates the inherent antimicrobial, antioxidant and cytocompatibility properties of these novel hydrogel dressings.

■ MATERIALS AND METHODS

Materials:

Gluconoacetobacter xylinus (ATCC 23770), *Pseudomonas aeruginosa* (NCIMB 8295) and *Staphylococcus aureus* (NCIMB 6571) were obtained from the University of Wolverhampton culture collection. *Candida auris* (NCF 8971) was obtained from the Public Health England Culture Collection, Porton Down, UK. A stock culture of *P. aeruginosa* and *S. aureus* were resuscitated at 37 °C on tryptone soy agar (TSA) (Sigma-Aldrich, UK), *G. xylinus* at 30 °C on mannitol agar and *C. auris* at 37 °C on Sabouraud Dextrose Agar (SDA). HS media was prepared following the standard protocol⁵¹. Material for mannitol agar, SDA and HS media were purchased from Lab M (UK). Tryptone soya broth (TSB), disodium phosphate and citric acid were purchased from Sigma-Aldrich (UK). Overnight broth cultures were prepared in appropriate medium using the stock plates.

U251MG (U251), MSTO and Panc 1 cell lines were purchased from ATCC (UK). Defibrinated horse whole blood was purchased from TCS Biosciences Ltd.

Silver nitrate was purchased from Fischer Scientific (UK). Hydroxypropyl- β -cyclodextrin (parenteral grade) was kindly provided by Roquette (France). Curcumin and Dimethyl sulfoxide (DMSO) (spectrophotometric grade) were purchased from Alfa Aesar (UK). Ringer tablets were purchased from Lab M (UK). Thiazolyl Blue Tetrazolium Bromide

(MTT), sodium bicarbonate and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (UK). Sodium chloride 0.9 % (w/v) (normal saline, intravenous infusion) was purchased from Baxter, UK. Sodium hydroxide was purchased from Acros Organics (UK). NaCl (5.8 g/L) and glycine (7.6 g/L) for preparing Sorensen's glycine buffer were purchased from Sigma-Aldrich, UK. Trypsin was purchased from Lonza (Belgium). Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), L-Glutamine and Antibiotic Antimycotic were purchased from Gibco (UK).

Preparation of CUR:HP β CD inclusion complex:

Inclusion complex was synthesised by the solvent evaporation method following the protocol previously reported²². Briefly, CUR solution (0.79 g in 37.5 mL acetone) was added dropwise to the aqueous HP β CD solution (3.0 g in 12.5 mL deionised water) under constant stirring at room temperature for slow and complete evaporation of acetone. The sample was centrifuged and the aqueous supernatant containing CUR:HP β CD inclusion complex was frozen after filtration and lyophilised.

Preparation and characterisation of cAgNP:

Preparation of cAgNP using CUR:HP β CD

A novel green chemistry approach was developed for cAgNP synthesis by reducing silver nitrate (AgNO₃) with aqueous solution of CUR:HP β CD. CUR:HP β CD (0.19 g) solution was prepared by dissolving in deionised water (18 ml). CUR:HP β CD aqueous solution was added drop-wise with constant stirring to 1 mM AgNO₃ aqueous solution (42 ml) under boiling condition in a conical flasks. The mixed solutions were boiled for 3 h for

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3 reduction of Ag ions followed by cooling at room temperature for 30 minutes. The flask
4 was covered with aluminium foil to maintain dark conditions throughout the reduction
5 process to avoid any photochemical reactions.
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10 11 12 **Characterisation of cAgNP:**

13 14 **Dynamic Light Scattering (DLS) and Zeta potential**

15 DLS measurements were performed on a Malvern Zetasizer (nano ZS) at the University
16 of Birmingham. Five consecutive measurements were carried out at 25 °C with samples
17 equilibrated for 2 minutes before the measurements were started. The results were
18 averaged to calculate the mean size. The same instrument was used to obtain the Zeta
19 Potential values. The same samples were used for size measurements, equilibrated for 2
20 minutes before measurements were started. The results were obtained at 25 °C. Five
21 consecutive measurements were taken and averaged to calculate the zeta potential.
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32 33 34 **Transmission Electron Microscopy (TEM)**

35 The shape, size and distribution of cAgNP produced using CUR:HP β CD was investigated
36 by TEM. Briefly, a drop of aqueous colloidal cAgNP was casted on a carbon-coated 300
37 mesh copper grid (agar scientific), left for 30 minutes, rinsed off and allowed to dry at
38 room temperature in a covered container. TEM imaging was performed using JEOL 1400
39 electron microscope, operated at 80 keV. Images were captured at different range of
40 magnifications. A 100 cAgNP were randomly selected and measured using ImageJ to
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Preparation of cAgNP-loaded BC hydrogels

Production of bacterial cellulose hydrogels

Bacterial cellulose (BC) hydrogels were prepared by the protocol reported previously²². Briefly, *G. xylinus* was selected for BC production and hydrogels were biosynthesised in sterilised Hestrin and Schramm (HS) culture medium under static condition. BC pellicles were purified prior to experimental use.

Loading cAgNP in BC hydrogels

BC pellicles after purification were padded dry on sterilised filter paper and aseptically loaded with cAgNP by immersing in aqueous colloidal cAgNP overnight under agitated conditions at 4 °C.

Characterisation studies:

Morphological study by Scanning electron microscopy (SEM):

Morphology of BC (neat) has been previously reported^{22,52}. BC before purification, purified BC (neat) and cAgNP-loaded BC hydrogels were cut in to discs (8 mm) using a biopsy punch and lyophilised. These discs were then coated with ultrafine gold coating using SC500 fine coater (Emscope, Kent, UK) and placed on a carbon stub. The morphology of the samples was studied using Zeiss Evo 50 EP, SEM (Carl Zeiss AG, Oberkochen, Germany).

Energy dispersive X-ray (EDX) analysis:

Lyophilised cAgNP-loaded BC hydrogel samples were analysed by EDX (Zeiss Evo 50 EP, SEM) coupled with X-Max^N 50 Silicon Drift Detector (Oxford Instruments) and the analysis was performed by Oxford Instruments INCA Energy Dispersive Spectroscopy Nanoanalysis software using the Point & ID function, to confirm the presence of silver.

Moisture content (M_c)

The wet mass (W_w) of BC (neat) and cAgNP-loaded BC (test) hydrogels was recorded before lyophilisation and the dry mass (W_d) was measured after 72 h lyophilisation. M_c (%) was calculated using a formula:

$$\% M_c = \frac{(W_w - W_d)}{W_w} \times 100$$

Cytocompatibility study (*in vitro* cell viability):

Cytocompatibility of BC (neat) has been previously tested and reported using conditioned media²⁰ and BC discs²² on different mammalian cell lines. To study the cytocompatibility of cAgNP-loaded BC, *in vitro* cell viability test was performed using Panc 1 (human pancreatic ductal adenocarcinoma), U251 (human glioblastoma) and MSTO (human mesothelioma) following the protocol previously reported²².

All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with fetal bovine serum (FBS), Antibiotic Antimycotic and L-Glutamine at

37 °C in humidity incubator with 5 % CO₂. BC pellicles after pad drying were either rehydrated with PBS (control) or cAgNP (test) overnight under agitated conditions (150 rpm) at 4 °C. These samples (control and test) were then aseptically cut in discs (8 mm) using a biopsy punch for experimental purposes and temperature adjusted to 37 °C before use.

Briefly, 25,000 cells were seeded per well (24 well plate) for 24 h at 37 °C in 5 % CO₂ incubator. The cells were exposed to either free cAgNP (equivalent amount) or cAgNP-loaded BC (8 mm discs) for 24 h to investigate the effect on cell viability. Cells without BC discs and PBS-loaded BC discs (8 mm) were used as controls respectively. Cell viability was evaluated following the standard MTT assay previously reported²². Data recorded was analysed statistically by two-way analysis of variance (ANOVA) with a Tukey's multi comparisons test using GraphPad Prism.

Haemocompatibility

The haemocompatibility of cAgNP-loaded BC hydrogels was tested following the protocol previously reported²². Briefly, defibrinated horse whole blood was washed twice using commercially available normal saline (pH 5.5) and blood cells were re-suspended in normal saline. cAgNP-loaded BC discs (8 mm) were cut using a biopsy punch and incubated with 1.9 ml horse blood cells suspended in normal saline in test Eppendorf tubes. The positive (+ve) control was blood cells suspended in distilled water and negative (-ve) control was blood cells in normal saline. All the test and control Eppendorf tubes were incubated at 4 °C for 2 h with periodic inversion (every 15 min). Post-

incubation, after the removal of BC discs from test Eppendorf tubes, all the tubes were centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was decanted and absorbance was recorded (540 nm) to calculate percentage haemolysis (% Haemolysis).

$$\% \text{ Haemolysis} = \frac{(\text{Abs of sample}) - (\text{Abs of } - \text{ve control})}{(\text{Abs of } + \text{ve control}) - (\text{Abs of } - \text{ve control})} \times 100$$

Antimicrobial study (Disc diffusion assay)

The antimicrobial activity of cAgNP-loaded BC was investigated against *P. aeruginosa* (Gram –ve), *S. aureus* (Gram +ve) bacteria and *C. auris* using the disc diffusion assay. PBS-loaded BC and HPβCD-loaded BC were used as controls. Discs (8 mm) of PBS-loaded BC, HPβCD-loaded BC and cAgNP-loaded BC were placed aseptically on TSA plates spread with an overnight culture of *P. aeruginosa* or *S. aureus* and SDA plates were spread with an overnight culture of *C. auris* and incubated at 37 °C for 24 h and zone of inhibition (ZOI) were measured. Data is presented as mean ± standard deviation (SD) and analysed statistically by two-way ANOVA with a Tukey's multi comparisons test using GraphPad Prism.

Anti-oxidant study by DPPH assay

The antioxidant potential of cAgNP produced using CUR:HPβCD was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The assay mixture with 1 ml DPPH (80 µg/mL) methanolic solution and 1 ml of test colloidal cAgNP (including

the blank) after mixing were incubated for 30 min in dark at the room temperature. Absorbance was recorded (517 nm) and the free radical scavenging potential was calculated as percent antioxidant effect (% E) as:

$$\% E = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

Transparency test

Monitoring the healing process without the need of removing dressing could help minimise trauma to the granulating tissue. With the aim of wound dressing application, the transparency level of cAgNP-loaded BC hydrogels was assessed by simulation. PBS-loaded BC and cAgNP-loaded-BC hydrogels were transferred on the laminated sheet of paper with text typed in different colours. The clarity of text underneath the hydrogel was examined to determine if the healing tissue at the wound site could be observed through these hydrogels.

■ RESULTS AND DISCUSSION

Preparation of cAgNP and cAgNP-loaded BC hydrogels

Most of the AgNP synthesis methods involve the use of organic solvents and toxic reducing agents with the potential threat to the environment and cytotoxic effect on mammalian cell lines. The method reported herein involved bio-reduction of AgNO₃ with an aqueous suspension of CUR:HPβCD resulting in nanoparticle formation. The colour changed from colourless to pale yellow at the start of CUR:HPβCD addition which intensified with increased dosage and subsequently changed to yellow-brown with time, suggesting the production of nanoparticles. This could be due to the excitation of surface plasmon vibrations in AgNPs, which is in accordance with literature^{40,53}.

G. xylinus produced BC hydrogel pellicles which were harvested and purified. BC pellicles became clear after purification. These results are in accordance with previous reports^{15,22,33,52,54}. When the purified padded dry BC was rehydrated with the aqueous colloidal cAgNP, the pellicles re-swelled and the colour changed to yellow-brown. These cAgNP-loaded BC hydrogels were stored at 4 °C throughout the experimental procedure.

Characterisation of cAgNP and cAgNP-loaded BC hydrogels

The formation of colloidal cAgNP produced was confirmed by testing its properties like size distribution and charge. After loading aqueous colloidal cAgNP in the padded dry BC, the physicochemical and *in vitro* haemocompatibility and cytocompatibility hydrogels were investigated.

Particle size distribution and surface charge by TEM, DLS and zeta potential

The size and morphology of cAgNP produced using CUR:HP β CD were studied from the TEM images (Fig. 1a-c). TEM imaging showed that the morphology of the nanoparticles was mainly spherical in shape with smooth edges. The majority of bio-reduced cAgNP were in the diameter range of 20-55 nm (80 %) and the average size of 42.71 ± 17.97 nm. (Fig. 1a-c). It emerged from the results that nanoparticles were homogeneously surrounded by a thin layer of capping material. As the method employed in the current work only involved AgNO₃ and CUR:HP β CD, without the use of any organic solvents and additional capping agents, this suggests that CUR:HP β CD was acting both as a reducing and capping agent. These results are in agreement with literature reporting the use of curcumin as a reducing and capping agent^{40,43,46,48,53}.

DLS measurements identified nanoparticles with the hydrodynamic diameter in the range of 182.10 ± 8.83 nm (Fig 1d) and the Poly dispersity index (PDI) value of 0.196 ± 0.009 . These results indicate both, the nucleation to form new nanoparticles and aggregation could be happening consecutively. These findings are consistent with studies reported in literature^{53,55}. Low PDI indicating that the colloidal cAgNP was not very polydispersed.

The zeta potential value is directly proportional to the stability of nanoparticle dispersion⁵⁵. Zeta potential studies revealed a negative charge on the synthesised nanoparticles with the magnitude of -20.1 ± 0.702 mV. These findings are in a close range to the previously reported values for AgNP bio-reduced using curcumin⁴³.

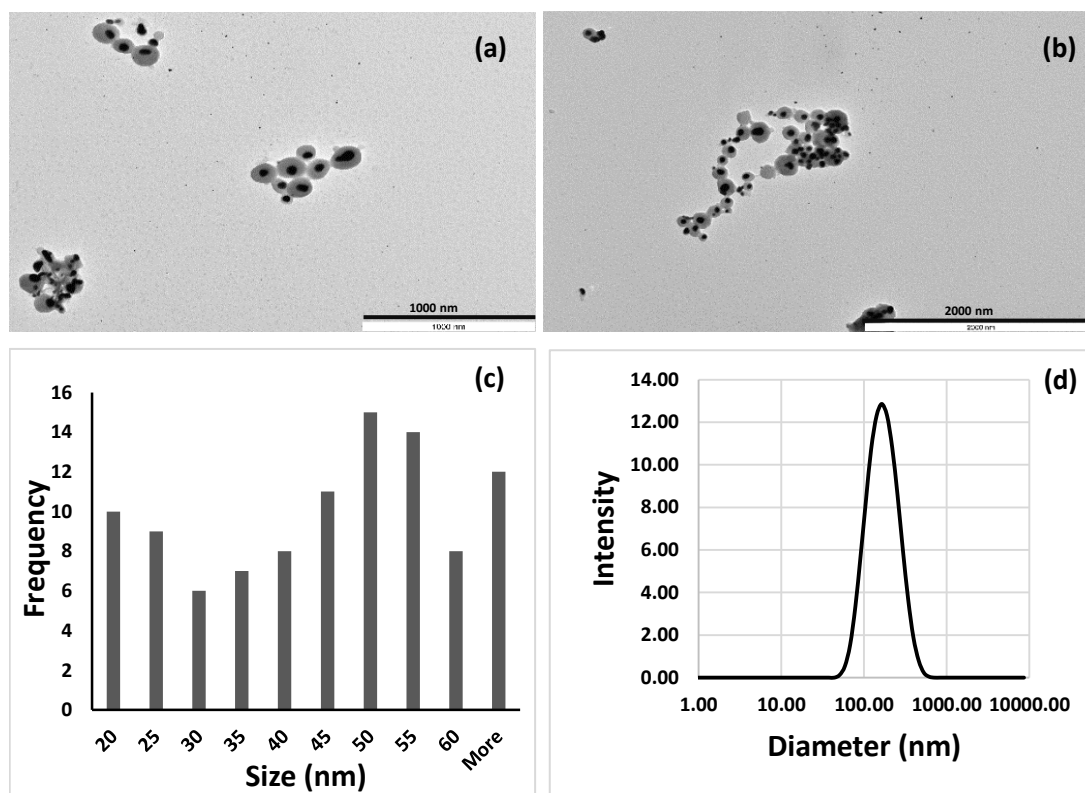


Figure. 1. Characterisation of cAgNP produced using CUR:HP β CD, (a-b) TEM photomicrographs (c) size distribution as measured by TEM analysis and calculated with 100 nanoparticles (d) DLS data of cAgNP with size distribution.

Scanning electron microscopy (SEM)

BC has a fine fibre network structure with voids^{21,22,52}. SEM images revealed *G. xylinus* trapped in the cellulose network before purification (Fig 2a) and bacteria were successfully removed on purification (Fig 2b). The interspersed voids in the interwoven cellulose ribbons of BC allow impregnation of healing agents^{17,33,56}. SEM of lyophilised cAgNP-loaded BC revealed that cAgNP penetrated through the voids during rehydration of padded dry BC pellicles and got physically trapped in the fibre network structure (Fig 2c). Also, it emerged that along with bio-reduced cAgNP, there was free CUR:HP β CD trapped in the BC. In addition to the reducing and capping properties in cAgNP synthesis, CUR:HP β CD has been demonstrated to have wound healing properties²². The excess of CUR inclusion complex in cAgNP-loaded BC may deliver additional benefits contributing to wound healing.

Morphology and size of nanoparticles was also determined in the SEM photomicrographs (Fig 2d). The average diameter was in the range of 37.2 - 65.1 nm and most of the nanoparticles appeared spherical. These results are in accordance with TEM results discussed in section above.

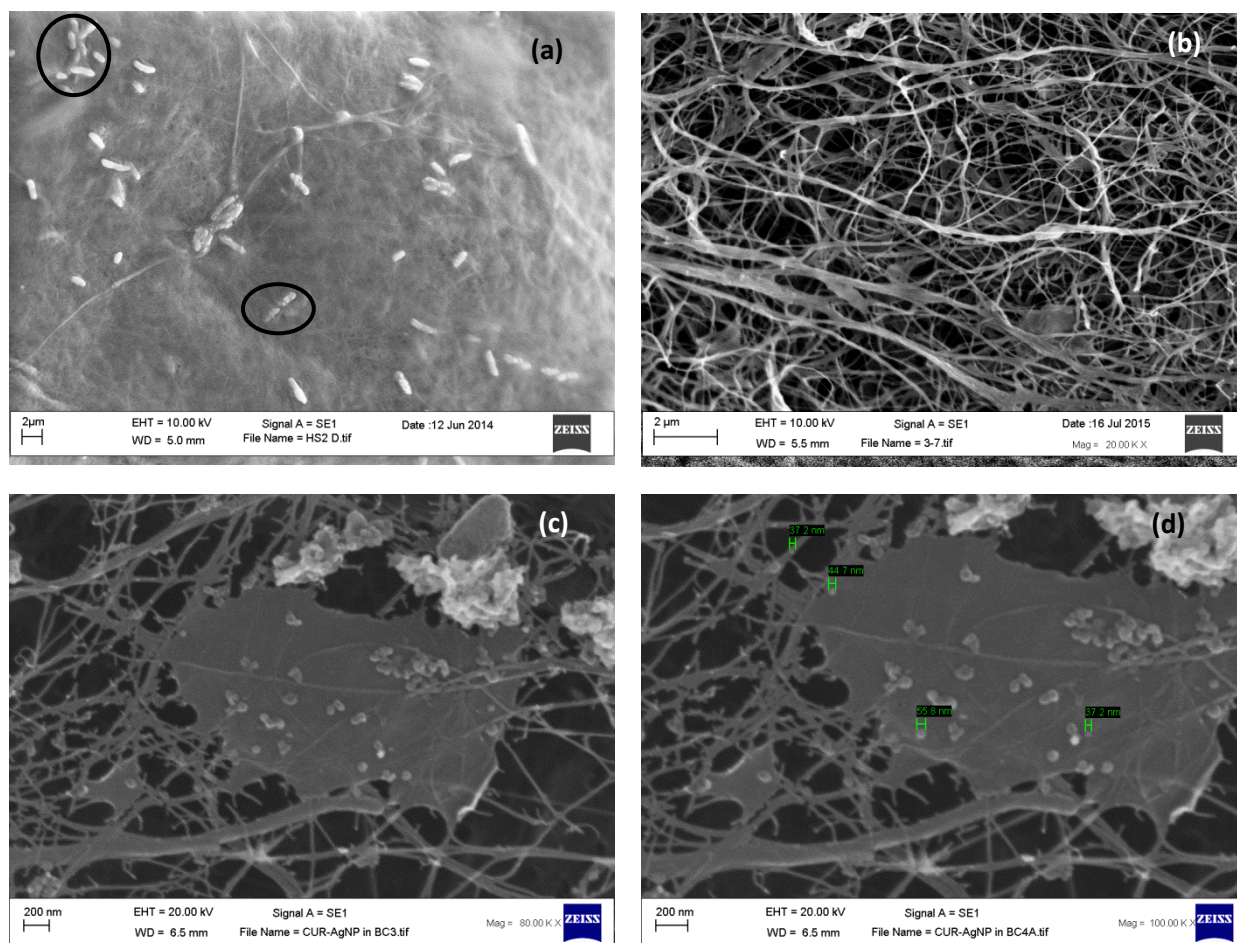


Figure 2. SEM photomicrographs of (a) Unwashed BC, entangled *G. xylinus* highlighted, (b) BC after purification, (c) & (d) cAgNP loaded in BC fibre network.

Energy dispersive X-ray (EDX) analysis

The elemental analysis was carried out by EDX studies. The results confirmed that BC (neat) is composed mainly of carbon and oxygen which is in accordance with our previously reported data³³. In addition to carbon and oxygen, detection of silver in the EDX spectra of cAgNP-loaded BC (test) confirmed nanoparticle loading in the test samples (Fig. 3).

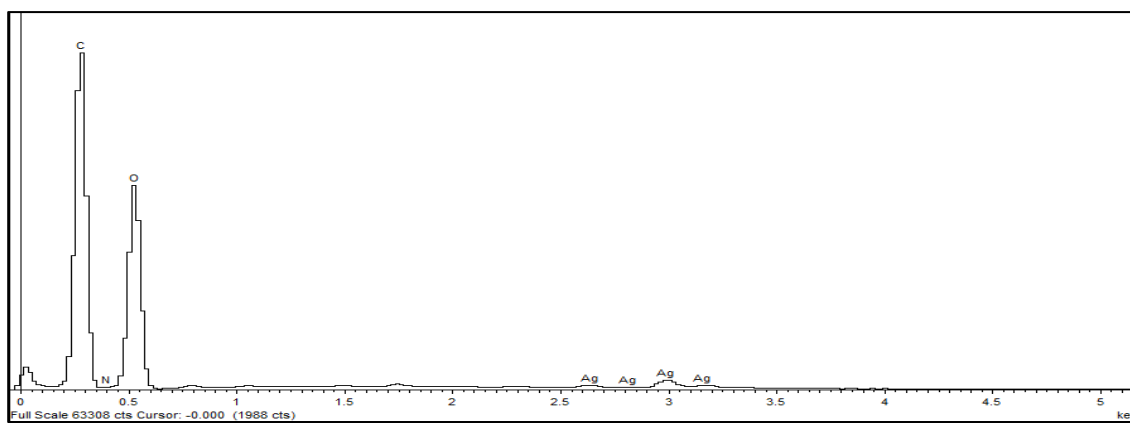


Figure. 3. EDX spectrum of cAgNP-loaded BC.

Moisture content (M_c)

BC hydrogels have been reported to have a unique property of resistance to degradation despite considerable moisture content which is several times its dry mass¹⁴. This strength is contributed by its cross-linked fibre network structure. In the current study, the moisture content of neat BC and cAgNP-loaded BC hydrogels was evaluated. The results confirmed that neat BC pellicles imbibed 99.68 ± 0.09 % (v/w) ($n = 3$) water which is in accordance with previously reported data (> 99.5 %) for neat BC hydrogels^{20,22}. Moreover, the study on cAgNP-loaded BC revealed that the moisture content in these hydrogels was 98.86 ± 0.04 % ($n = 3$). These findings indicate that the difference in the

mass of neat BC and cAgNP-loaded BC is contributed by cAgNP and CUR:HP β CD that gets physically trapped in the fibre network of BC during the loading process.

The high moisture content in BC can confer many benefits such as increased malleability, soft texture and creating a moist environment with increased dissolved oxygen to facilitate aerobic conditions at the wound-dressing interface. In addition, these hydrogels may ease removal of the dressing, reduce pain sensation and therefore improve patient comfort. These features have been reported to facilitate the wound healing process^{15,21,57,58} and therefore BC has attracted increased interest in the wound care sector.

Cytocompatibility (*In vitro* study)

Cytocompatible nature of BC is well documented in literature^{20,21,22,59}. It is one of the many intrinsic features that resulted in BC based proprietary products like DermafillTM, Biofill[®], XCell[®], and Gengiflex[®]^{57,60-62}. In this study, the cytocompatibility of cAgNP-loaded BC hydrogels was evaluated using three different mammalian cell lines. The cytotoxicity as determined by MTT assay revealed that cAgNP-loaded BC is cytocompatible as all the tested cell lines demonstrated good survival rate (Fig. 4a).

Furthermore, we compared the cytocompatibility of free cAgNP to cAgNP-loaded BC on U251, MSTO and Panc 1 cell lines. The results demonstrated that free cAgNP had cytotoxic effect on all the tested cell lines resulting in lower cell viability compared to cAgNP-loaded in BC ($p < 0.001$) (Fig. 4 a,b). These results suggest that BC controls the

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release of cAgNP thus minimising the cytotoxic effect on the mammalian cells. These findings support the potential application of cAgNP-loaded BC for wound management as hydrogel dressings.

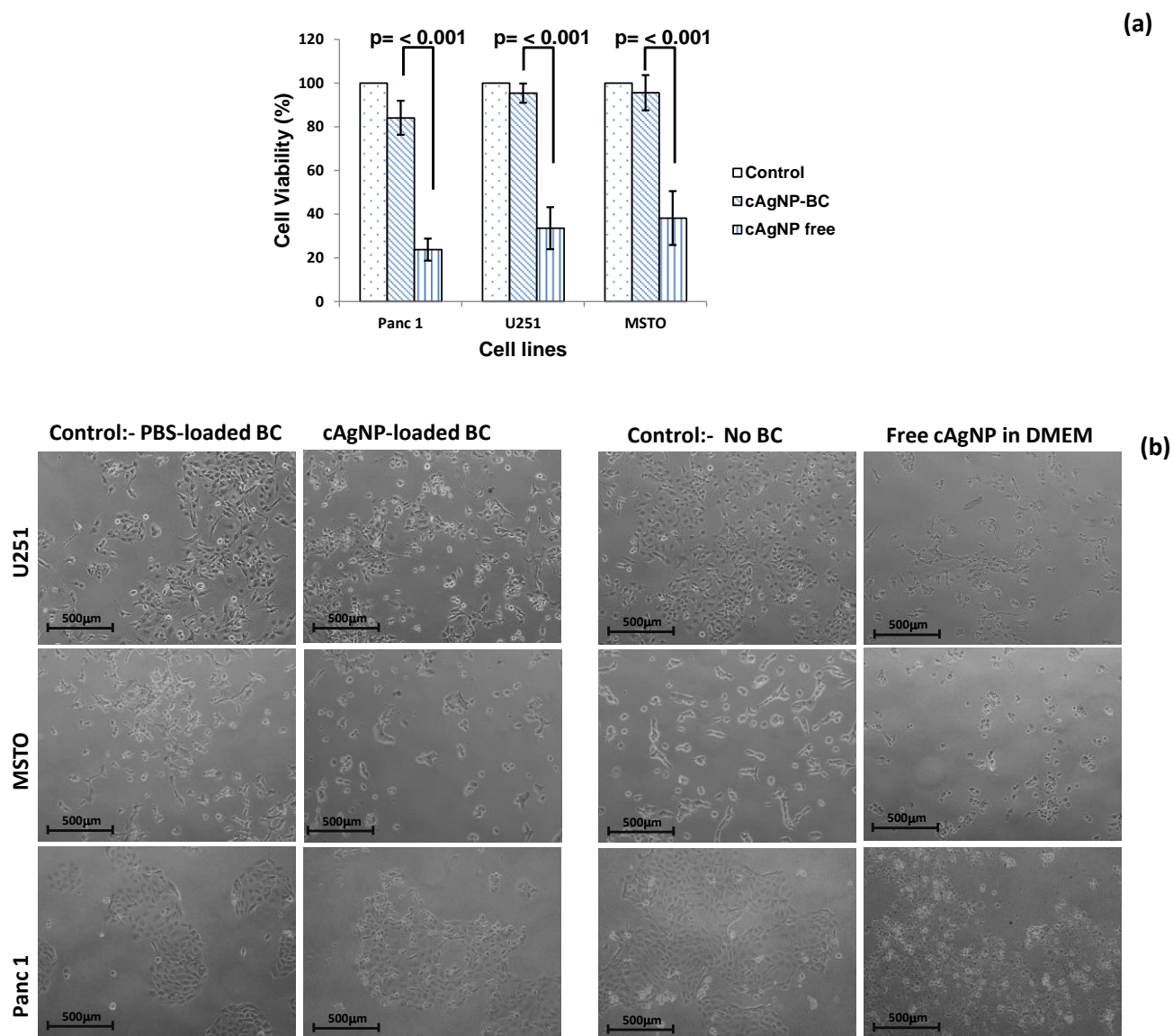


Figure. 4. Cytocompatibility test results. (a) Bar graph showing the cell viability (%) after 24 h exposure to cAgNP-loaded BC and free cAgNP (equivalent amount) ($n = 8$). Representative photomicrographs of cells (10x magnification) after exposure to cAgNP-loaded BC and free cAgNP for 24 h.

Haemocompatibility

Haemocompatibility is an important property for biomedical applications of a material. BC has been reported to be haemocompatible hence it has been used in proprietary wound dressings^{20,63,64}. In the current study, cAgNPs were prepared in deionised water hence the hypothesis was drawn that cAgNP-loaded BC hydrogels may have haemolytic properties. The test results revealed that cAgNP-loaded BC hydrogels have a percentage haemolysis of 6.85 ± 1.12 % (n = 6). According to the ASTM F756 standards haemolytic indices this range is over the threshold value of 5 % hence cAgNP-loaded BC hydrogels would be classified as a haemolytic material⁶⁵. The higher haemolysis (%) of the tested hydrogels could be attributed to the use of deionised water instead of the isotonic solution for the synthesis of cAgNP.

In the case of chronic wound, there could be necrotic tissue or slough at the wound bed hence the haemolytic behaviour of these hydrogels may be minimal. Further research on the production of AgNP using CUR:HP β CD dissolved in isotonic solution may improve haemocompatibility of these hydrogels.

Antimicrobial study

Invasion of opportunistic microbes could impair wound healing leading to chronic non-healing wounds^{6,66}. Silver nanoparticles have been intensively studied as antimicrobial agents^{40,49,53}. Different mechanisms of action of AgNPs have been proposed as their antibacterial and antifungal effect is not completely known⁶⁷. In bacteria AgNPs have the ability to increase the permeability of cell membrane, interfere with DNA replication,

denaturation of bacterial proteins and release of silver ions inside the bacterial cell^{49,67}.

The antifungal action of silver nanoparticles are thought to increase the reactive oxygen species within the fungal cell and increase membrane permeability resulting in cell death⁶⁸.

In the current study, PBS-loaded BC, HP β CD-loaded BC and cAgNP-loaded BC hydrogels were tested against *P. aeruginosa*, *S. aureus* and *C. auris* using the disc diffusion assay at 24 h. PBS-loaded BC and HP β CD-loaded BC did not exhibit antimicrobial activity however, cAgNP-loaded BC demonstrated significant antimicrobial activity ($p < 0.001$) against all three of the tested microbial strains (Fig. 5).

These results confirm the broad spectrum antimicrobial activity of cAgNP-loaded in BC. Moreover, it is observed that cAgNP-loaded BC have stronger antimicrobial activity (Fig 5) against *P. aeruginosa* as compared to *S. aureus* ($p < 0.001$). This could be explained by the difference in the cell structure of Gram positive and Gram negative bacteria. AgNPs have been reported to have the ability to separate cytoplasm from bacterial cell wall (plasmolysis effect) leading to the cell death in *P. aeruginosa*. In *S. aureus*, AgNPs act differently and cause the bacterial cell death by inhibiting the cell wall synthesis^{49,67,69}.

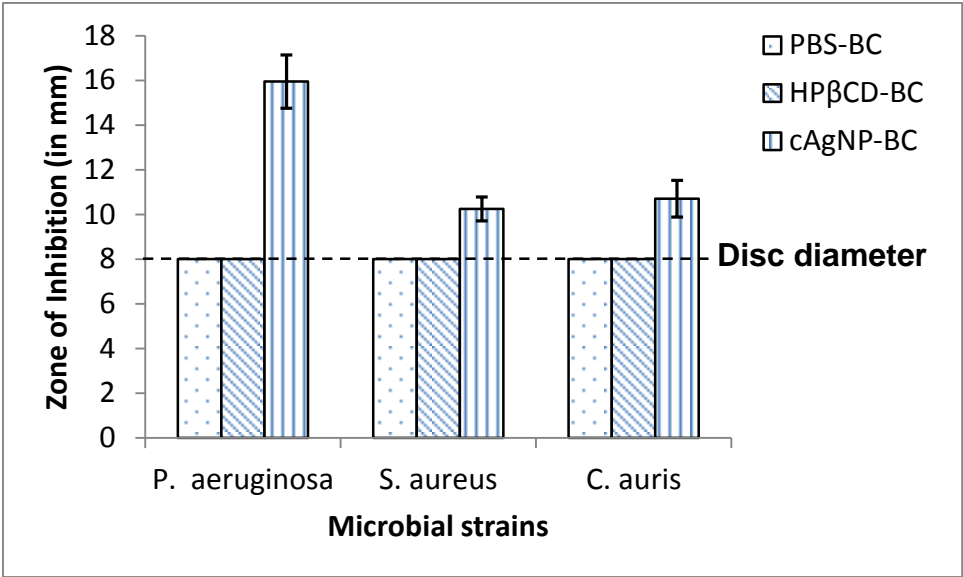


Figure. 5. Antimicrobial disc diffusion assay results for PBS-loaded BC, HPβCD-loaded BC and cAgNP-loaded BC against *P. aeruginosa*, *S. aureus* and *C.auris* at 24 h (n = 10; error bars = SD).

Anti-oxidant activity of cAgNP by DPPH assay

Oxidative stress at the wound site may interfere with the healing process⁷⁰ hence a wound dressing with antioxidant properties along with antimicrobial activity may prove beneficial⁷¹. CUR has been reported to have healing properties including antimicrobial and antioxidant activities. It has been previously demonstrated that these properties were preserved in the inclusion complex of CUR in HPβCD²².

In the current study, cAgNP were successfully prepared using CUR:HPβCD as reducing and stabilising agent. The percent antioxidant effect (% E) for aqueous cAgNP colloidal suspension against DPPH was determined to be 76.65 ± 3.21 % (n = 6). The test results confirmed that silver nitrate and HPβCD does not have antioxidant activity which is in

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3 accordance with previously reported findings²². These results confirmed that colloidal
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5 cAgNP aqueous medium produced using CUR:HP β CD has antioxidant activity and when
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7 loaded in BC to produce hydrogels, could prove beneficial in wound healing process.
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17 Monitoring of wound healing process is vital from a clinical perspective⁷²⁻⁷⁴. Routinely,
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19 this involves the removal of a dressing which can disturb the granulating tissue and could
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21 cause trauma to the wound. A dressing with the feature enabling non-invasive monitoring
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23 could be beneficial in wound healing.
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27 The transparent nature of BC has already been reported²² and in this study, PBS-loaded
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29 BC hydrogels reconfirmed these findings (Fig 6a). In this study, the transparency
30
31 property of cAgNP-loaded BC was evaluated by reading the text in different colours on
32
33 white laminated paper sheet through the test hydrogels. The results (Fig. 6b) demonstrate
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35 cAgNP-loaded BC hydrogels allow monitoring the wound without the need for removal
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37 of the dressing. This transparency testing is a simulation study and in order to confirm the
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39 results, clinical validation would be required and could be evaluated using *in vivo* animal
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41 models.
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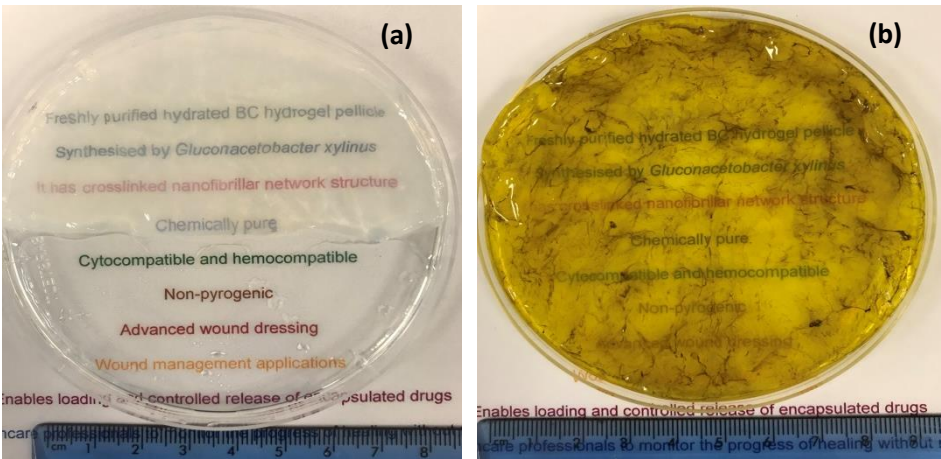


Figure. 6. Photomicrographs with the visual appearance of (a) BC loaded with PBS (b) cAgNP-loaded BC hydrogel pellicle.

■ **CONCLUSION**

The development of advanced polymeric materials requires precise evaluation of structure, properties and behaviour to avoid potential failures of the commercial products manufactured from them. The current study demonstrates the production, physicochemical and *in vitro* characterisation of cAgNP-loaded BC hydrogels. It also increases efficiency and minimizes the potential failure of precisely structured biomaterials before, during and after specific applications. The results confirmed that cAgNPs were successfully synthesised, following the green chemistry approach using CUR:HP β CD and loaded in BC to produce hydrogels with a potential wound dressing application. These hydrogels demonstrated broad-spectrum antimicrobial activity along with antioxidant properties. Moreover, the hydrogels showed cytocompatibility with the tested cell lines. The high moisture content and the good level of transparency further advocate their potential application in the management of chronic wounds with high microbial bioburden.

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■ CONFLICT OF INTEREST

The authors confirm no conflict of interest. The authors alone are responsible for the content and writing of the article.

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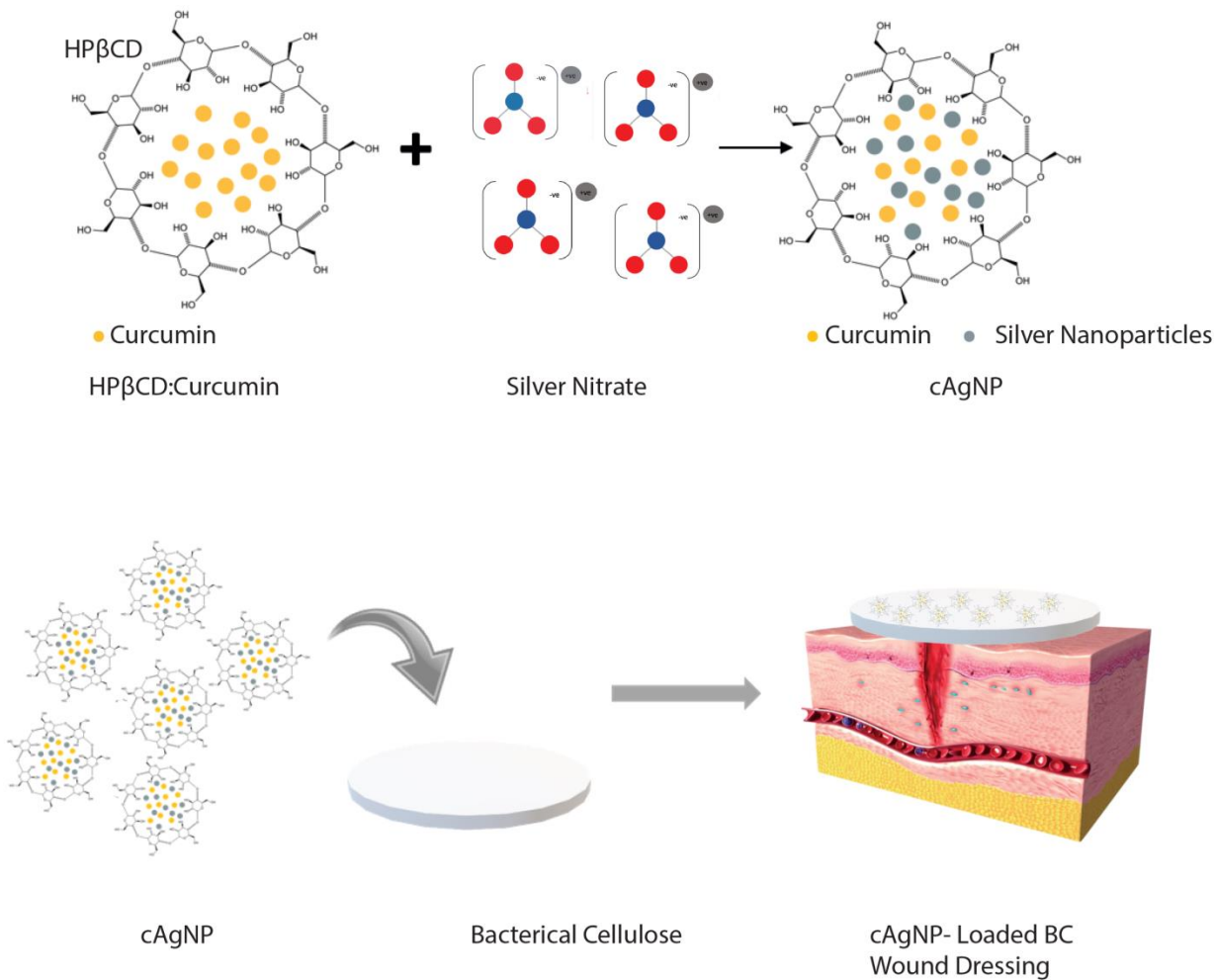
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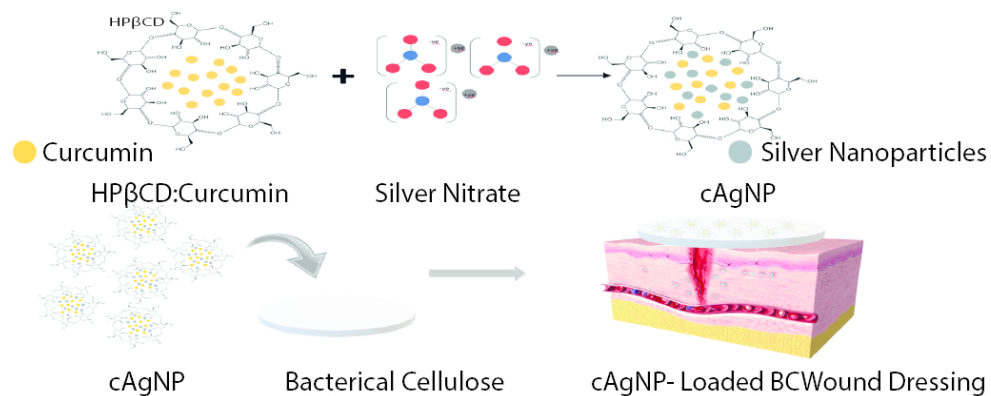
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Synthesis of silver nanoparticles using curcumin-cyclodextrins loaded into bacterial cellulose based hydrogels for wound dressing applications

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